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**13. Abstract** ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The gene of the nuclear receptor coactivator AIB1 is amplified in breast cancer cell lines as well as in breast tumor tissue. AIB1 mRNA is often highly expressed in primary breast tumors and has been shown that AIB1 enhances estrogen and progesterone dependent transcription in vitro. Therefore, it has been postulated that AIB1 contributes to the development of breast cancer. However, it is currently not known what the precise role of AIB1 is in the development of breast cancer. To address this question, we established MCF-7 breast cancer cell lines in which we can regulate AIB1 levels with ribozymes in order to determine the impact of reduced AIB1 gene expression on the phenotype and angiogenic or invasive properties of breast cancer cells. Here we report that depletion of endogenous AIB1 levels reduced steroid hormone signaling via the estrogen receptor-alpha or progesterone receptor-beta as well as estrogen-mediated inhibition of apoptosis and cell growth. Furthermore, we demonstrate that upon reduction of endogenous AIB1 expression, estrogen-dependent colony formation in soft agar and tumor growth of MCF-7 cells in nude mice was decreased. We have now also demonstrated that reduction in AIB1 levels significantly decreases the invasive and motile behavior of MCF-7 cells, in particular in the focal adhesion factor of the MCF-7 cells. We conclude that AIB1 exerts a rate-limiting role for hormone dependent human breast tumor growth.

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## Introduction

Small fat-soluble hormones, such as steroids, retinoids and vitamin $D_3$ play pivotal roles in the control of breast cancer proliferation and differentiation. The biological effects of these molecules are mediated through intracellular receptor proteins. Estrogens stimulate proliferation of estrogen receptor (ER) positive breast cancer cells and the ER status of a breast tumor is predictive of the outcome of the disease. Therapy (such as tamoxifen) targeted at reducing the estrogenic stimulus to the breast has been shown to be effective. Conversely, retinoids and vitamin $D_3$ are strongly growth inhibitory in breast tumor cells and analogues of these compounds are currently being tested for efficacy in breast cancer. Recently, the discovery of proteins known as steroid receptor coactivators has led to another level of complexity to our understanding of how hormones exert their effects (1,2). Of particular interest is the coactivator **AIB1 (amplified in breast cancer 1),** which interacts with the estrogen receptor (ER) (3). Interestingly, the AIB1 gene was found to be amplified and AIB1 expression is increased in breast tumors and breast cancer cell lines (3,4,5). **However, it is currently not known what the role of AIB1 amplification is in the development of breast cancer, nor is it known whether detection of AIB1 overexpression will be valuable for diagnosis of breast cancer or for prognosis of disease outcome.** The possible role of AIB1 in breast cancer is further complicated by studies that demonstrate that AIB1 can also interact wit retinoid, thyroid, $VitD_3$, PPAR and androgen receptors (6-8). **This broad ability to potentiate the effects of a number of hormone receptors leads to the question of what is the precise role of the AIB1 amplification in breast cancer.**

In this proposal we will investigate the hypothesis that overexpression of AIB1 in breast cancer is important for breast tumor development by impacting upon nuclear hormone receptor function. In particular, we wish to investigate if AIB1 is required for expression of a gene(s) critical for breast cancer development. The novel approach we will take to study this question will be to develop hammerhead ribozymes to target and cleave AIB1 thus production a selective reduction of this coactivator in breast cancer cells. Ribozymes are molecules of RNA that can cleave a specific target RNA (in this case AIB1) and thus selectively reduce expression of the AIB1 protein in the cell. In addition, the potential development of ribozymes as potent therapeutic agents (9) makes the translation of these results into possible therapies realistic. **In this study we will examine the idea that the nuclear receptor coactivator AIB1 is rate-limiting for breast cancer development and that selective targeting of this coactivator will be useful for future development of novel therapies leading to reduced proliferation or metastatic potential of these cells.**

Initially our experiments have been focused on designing ribozymes that selectively decrease AIB1 in vivo (see first Annual Report 2000). Using these reagents as tools, we will now determine the impact of reduced AIB1 gene expression on estrogen, progesterone, retinoid, Vit $D_3$, PPAR and AR receptor function in breast cancer cell lines. Finally, we will determine if reduction of AIB1 mRNA can influence the progression of breast cancer in vivo. **We anticipate that these experiments will give valuable insights into the biological significance of AIB1 as well as its potential role as a therapeutic target in breast cancer.**

**Proposal Body**

In the approved Statement of Work, three Tasks were outlined:

Task 1: Development of AIB1 selective targeted ribozymes (month 1-12)

Task 2: Determining the impact of reduction in AIB1 mRNA on the phenotype of breast cancer cells (6-30 month)

Task 3: To determine if the reduction in AIB1 mRNA alter the angiogenic or invasive properties of breast cancer cell lines (12-36 month).

**Task 1:** The first goal of our studies was the development of a series of plasmids that would specifically target AIB1 mRNA in vectors with the CMV promoter and also in vectors with the regulatable tetracycline promoter.

We completed Task 1 during the first year of the funding period. In summary, we were able to **a.)** design, construct and transfect several plasmids containing regulatable AIB1 ribozymes, and **b.)** to obtain stably transfected MCF-7 cell lines in which AIB1 protein levels were reduced by up to 90% compared to wild type cells (see first Annual Report from 2000). We have published results from this in List et. al. 2001 JBC.

**Task 2:** The goals of our studies regarding Task 2 were to **a.)** determine the impact of reduction of AIB1 mRNA on gross phenotype changes in cells including proliferation, differentiation and apoptosis and **b.)** to determine the effect of AIB1 reductions on gene expression and promoter activity of individual hormones.

**Rate-limiting role of AIB1 for MCF-7 breast cancer cell growth**

In order to analyze the influence of AIB1 on the phenotype of human breast cancer cells we performed growth assays and soft agar colony formation assays with MCF-7 cells expressing regulatable AIB1 ribozymes (development of these cell lines was described in the first Annual Report; see also Task 1). We found in cell proliferation assays that reduction of endogenous levels of AIB1 in MCF-7 breast cancer cells reduced estrogen-dependent growth of these cells (Appendix: Manuscript Fig. 4). In addition, estrogen-dependent colony formation of MCF-7 cells was strongly reduced after downregulation of AIB1 (Appendix: Manuscript Fig. 7). Furthermore, when we tested tumor growth of mCF-7 cells in nude mice we could demonstrate that reduction of AIB1 levels significantly decreased tumor growth of these cells in nude mice (Appendix: Manuscript Fig. 8). In summary, we were ably to demonstrate that the nuclear coactivator AIB1 exerts a rate-limiting role for hormone-dependent human breast cancer cell growth.

**Role of AIB1 for cell cycle progression and apoptosis in MCF-7 cells**

Since estrogens contribute to cell cycle progression and inhibition of apoptosis in MCF-7 cells, we analyzed whether slower estrogen-mediated growth after AIB1 downregulation might have resulted from a reduced ability of these cells to progress through the cell cycle or whether this effect might have been based on their altered susceptibility towards apoptosis. While we found no changes in cell cycle progression of MCF-7 cells after downregulation of AIB1 (Appendix: Manuscript Fig 5), we could demonstrate that the ability of these cells to inhibit apoptosis after estrogen stimulation was strongly reduced in cells with lower AIB1 levels, indicating that the reduction of estrogen-mediated cell growth of MCF-7 cells expressing the AIB1 ribozyme is at least partially due to the reduced ability of estrogen to inhibit apoptosis.

**Effect of AIB1 reduction on estrogen and progesterone responsive promoters**

Here we tested where AIB1 levels influenced the inducibility of estrogen and progesterone responsive promoters in MCF-7 cells. Our data showed that reduction of endogenous AIB1 levels in MCF-7 c ells lead to a reduction of estrogen and progesterone responsiveness of a estrogen/progesterone-responsive test promoter (Appendix: Manuscript Figs. 1 & 3). We could therefore demonstrate that AIB1 is necessary for full transcriptional activation of hormone-responsive promoters in vivo.

**Task 3:** The goals of our studies were to determine if the reduction of AIB1 mRNA alter the angiogenic or invasive properties of breast cancer cell lines. We have used the ribozyme cell lines to examine motility and invasive behavior of MCF-7 cells. (see figure below). We next determined if AIB1 was involved in other growth factor induced phenotypic changes and in particular in changes related to the invasive and motile behavior of tumor cells. To determine the role of AIB1/Δ3AIB1 in focal adhesion turnover of MCF-7 cells, total endogenous AIB1 was depleted using ribozyme targeting of endogenous AIB1 and the ability to form focal adhesions was tested in the presence of EGF, see figure below. Focal adhesions were identified with a monoclonal antibody against paxillin that specifically localizes to focal adhesion structures. These responses were characterized by rapid turnover of focal adhesions and reduction of AIB1 significantly attenuated these effects.

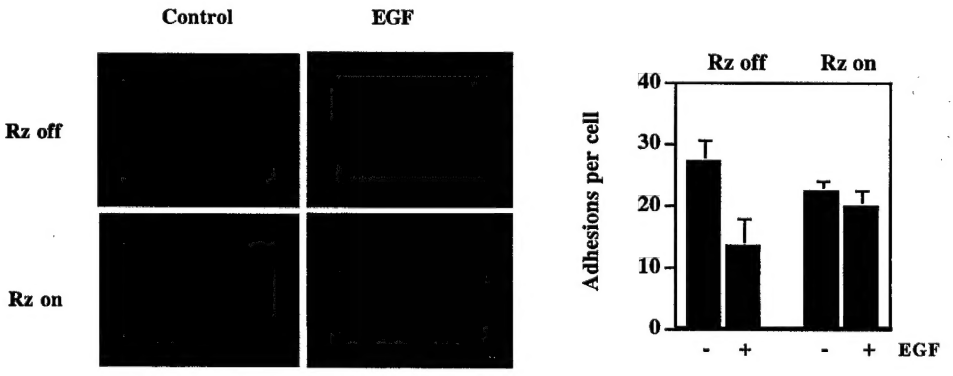


***Figure 1*** *– For these experiments we examined actin localization using fluorescein isothiocyanate (FITC) tagged phalloidin, a fungal toxin that exhibits specific F-actin binding capacity. Focal adhesions were identified with monoclonal antibody against paxillin, an FAK binding protein that specifically localizes to these adhesion structures. As expected in MCF-7 cells, EGF induces a rapid and profound remodeling of both actin cytoskeleton and focal adhesions. These responses were characterized by a rapid turnover of focal adhesions*

## Key Research Accomplishments

- We demonstrated that AIB1 is necessary for full transcriptional activation of the estrogen receptor alpha and the progesterone receptor beta in vivo.
- We showed that AIB1 is rate-limiting for estrogen-dependent cell growth in human MCF-7 breast cancer cells.
- We showed that downregulation of endogenous AIB1 levels in MCF-7 cells did not affect estrogen-stimulated cell cycle progression but reduced estrogen-mediated inhibition of apoptosis.
- We demonstrated that upon reduction of endogenous AIB1 expression, estrogen-dependent colony formation in soft agar and tumor growth of MCF-7 cells in nude mice were decreased.
- We demonstrated that ribozyme targeting of nuclear coactivator AIB1 reduces estrogen-dependent proliferation and neoplastic growth.
- We showed that other members of the nuclear receptor coactivator p160/SRC family cannon compensate for the loss of AIB1.
- We demonstrated that overexpression of AIB1 provides a selective advantage for tumor growth in mammary epithelium.

## Reportable Outcomes

List, H-J., Lauritsen, K.J., Reiter, R., Powers, C., Wellstein, A., and Riegel, A.T. (2001). Ribozyme targeting demonstrates that the nuclear receptor coactivator AIB1 is a rate-limiting factor for estrogen-dependent growth of human MCF-7 breast cancer cells. *J. Biol. Chem.* 276, 23763-23768.

List, H-J., Oh, A., Mani, A., Bowden, E.T., Reiter, R., Wellstein, A., and Riegel, A.T. The nuclear receptor coactivator AIB1 mediates insulin-like growth factor 1 and heregulin-induced proliferation of human MCF-7 breast cancer cells. Manuscript in preparation.

List, H-J., Oh, A., Mani, A., Bowden, E.T., Reiter, R., Wellstein, A., and Riegel, A.T. The nuclear receptor coactivator AIB1 mediates insulin-like growth factor 1 and heregulin-induced proliferation of human MCF-7 breast cancer cells. Abstract for poster presentation at the Era of Hope meeting in Orlando, FL September 2002. (See Appendix for poster example).

## Conclusions

Human breast tumorigenesis is promoted by the estrogen receptor pathway, and nuclear receptor coactivators are thought to participate in this process. Here we studied whether one of these coactivators, AIB1 (amplified in breast cancer 1), was rate-limiting for hormone-dependent growth of human MCF-7 breast cancer cells. We developed MCF-7 breast cancer cell lines in which the expression of AIB1 can be modulated by regulatable ribozymes directed against AIB1 mRNA. We found that depletion of endogenous AIB1 levels redued

steroid hormone signaling via the estrogen receptor α or progesterone receptor β on transiently transfected reporter templates. Down-regulation of AIB1 levels in MCF-7 cells did not affect estrogen-stimulated cell cycle progression but reduced estrogen-mediated inhibition of apoptosis and cell growth. Finally, upon reduction of endogenous AIB1 expression, estrogen-dependent colony formation in soft agar and tumor growth of MCF-7 cells in nude mice was decreased. From these findings we conclude that, despite the presence of different estrogen receptor coactivators in breast cancer cells, AIB1 exerts a rate-limiting role for homone-dependent human breast tumor growth.

In conclusion, under this grant Tasks 1 and 2 have been completed and published in a high impact scientific journal. Task 3 is substantially completed and data from this will be included in a manuscript that is currently in preparation.

**References**


1. Horwitz, K.B., Jackson, T.A., Bain, D.L., Richer, J.K., Takimoto, G.S. and Tung, L. Nuclear receptor coactivators and corepressors. *Mol. Endocrinology.* 10:1167-1177, 1996.

2. Glass, C.K., Rose, D.P. and Rosenfeld, M.G. Nuclear receptor coactivators. *Curr. Opin. Cell Biol.* 9:222-232, 1997.

3. Anzick, A.L., Kononen, J., Walker, R.L., et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 277:965-968, 1997.

4. Guan, X.Y., Xu, J., Anzick, S.L., Zhang, H., Trent, J.M. and Meltzer, P.S. Hybrid selection of transcribed sequences from microdissected DNA: isolation of genes within an amplified region at 20q11-13.2 in breast cancer. Canc. Res. 56:3446-3450, 1996.

5. List, H-J., Reiter, R., Singh, B., Wellstein, A., and Riegel, A.T. Expression of the nuclear coactivator AIB1 in normal and malignant breast tissue. *Breast Cancer Res. Treat.* 68:21-28, 2001.

6. Chen, H., Lin, R.J. Schlitz, R.L., et.al. Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell 90:569-580, 1997.

7. Li, H., Gomes, P.J. and Chen, J.D. rAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. Proc. Natl. Acad. Sci USA 94:8479-8484, 1997.

8. Takeshita, A., Cardona, G.R., Koibuchi, N., Suen, C-S., and Chin, W.W. TRAM-1, a novel 160-kDa thyroid hormone receptor activator molecule, exhibits distinct properties from steroid receptor coactivator-1. J. Biol. Chem. 272:27629-276334,

1997.

9. Czubayko, F., Malerczyk, C. and Wellstein, A. Synthetic anti-pleiotrophin ribozymes are effective treatment for metastatic melanoma in mice. In progress, 2002.